# Use of large-volume, fixed-depth, disposable slides for post-vasectomy semen analysis

P. HANCOCK*, K. LINDSAY† and M. T. TOMLINSON‡

*Andrology Laboratory, Yeovil District Hospital, Yeovil, Somerset;
†Andrology Department, Imperial Healthcare, Hammersmith Hospital, London; and
‡Andrology Department, Nottingham University Hospital, Derby Road, Nottingham

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**ABSTRACT**

Vasectomy is the surgical procedure used for male contraception. Traditionally, operative success has been established by the issue of a laboratory report stating the achievement of azoospermia. The purpose of this study is to establish if this is an achievable or realistic status and if a change to the current best practice would provide an acceptable and cost-effective alternative. In principle, human fecundity is complex and measured in probabilities, which is inconsistent with the implied absolute requirement to establish the complete absence of spermatozoa.

KEY WORDS: Analysis.
Andrology.
Semen.
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## Introduction

Vasectomy is one of the most common worldwide procedures for effective contraception, and it remains safe, cheap, effective and reliable.[1] Post-vasectomy semen analysis (PVSA) has become the standard for establishing operative success, with or without histological examination of vas deferens being a routine part of the procedure. The prime reason for PVSA is both to confirm the clearance of sperm and also to establish either technical surgical failure or early recanalisation, which may not be possible by examination of the resected portion of the vas deferens. Debate continues to try to establish the ideal time and number of samples for post-vasectomy analysis to ensure both clearance and patient compliance along with current best practice.[2] However, results depend on frequency of sexual activity and patient age, which produce considerable person-to-person variance in terms of the time period.[3,4]

The assessment of low numbers of sperm in semen causes a number of problems in the routine laboratory. These problems are associated with the detection of numbers of sperm below valid detection limits when test precision is taken into consideration. Currently, the only evidence-based method that has undergone peer review is that recommended by the British Andrology Society (BAS) guidelines for PVSA,[5] published in 2002. However, these guidelines and publications from many other workers state that the protocols were there to establish azoospermia. The World Health Organization (WHO) manual for the examination of human semen[6] states that "only when no spermatozoa are found after a complete and systematic search of all the resuspended precipitate should samples be classified as azoospermic". However, it is the belief of many that a complete and systematic search of the entire ejaculate may be neither cost-effective nor necessary.

In normal practice, azoospermia remains a description of the ejaculate; for example, "nil sperm seen" rather than a statement of its origin or a basis for diagnosis or therapy. All analytical methodologies have a limit of detection which is dependent upon the imprecision at the lower end of the analytical range. Observing single or occasional spermatozoa will in all likelihood be below the statistically derived limit of detection. This means that while we may be confident that in such circumstances azoospermia has not been achieved when no sperm are seen, the same confidence in a numerical result is lacking on the basis that if we looked harder we might find an elusive sperm. Essentially, it is impossible to determine a zero value with statistical plausibility.

In 2006, Cooper *et al.*[7] raised this point with the publication of data that demonstrated the potential presence of sperm even when none was observed in laboratory analysis. In this study, a trial of large-volume slides with fixed coverslips was compared with the traditional systematic examination of a 'drop' or a typical 10 µL semen on a glass slide covered with

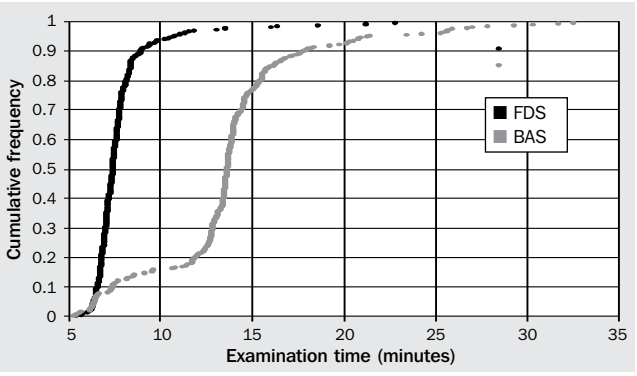


**Fig. 1.** Time taken to perform systematic x–y examination of a post-vasectomy semen sample using the traditional BAS method of a 22x22 mm slide of ~10 µL semen combined with an examination of the centrifuged deposit compared with a single-examination FDS holding 25 µL.

*Correspondence to Paul Hancock*
*Email: paul-hancock@hotmail.co.uk*

a 22 x 22 mm coverslip producing an approximate chamber depth of 20 µm.

Therefore, in reviewing the current BAS guidance after 10 years of implementation, a number of pertinent questions were raised:

- If azoospermia is not a realistic diagnosis for the laboratory to achieve, should the definition of a successful vasectomy be defined by some other standard?
- If the uncertainty surrounding the diagnosis of azoospermia is so high, could success be defined by a method other than those recommended by WHO or previously by BAS?

To answer the second point, a preliminary investigation was undertaken to establish whether a method could be developed using a large, fixed-volume slide (25 µL) and shown to be as sensitive as the method which currently employs an initial evaluation of 10 µL followed by a pellet examination as standard. From this initial data, a trial was undertaken in three different centres to determine whether the large-volume slide method could be viewed as interchangeable with the current standard.

**Table 1.** Pilot results identifying spermatozoa from placing a drop of semen from post-vasectomy patients on a glass slide and examined by direct light microscopy with a systematic x–y pattern over a 22x22 mm cover slip (BAS recommended).

| | Number of samples | % |
|---|---|---|
| No sperm seen | 169 | 82.8 |
| ≥1 sperm | 30 | 14.7 |
| Too many sperm to count | 2 | 1.0 |
| Motile sperm | 3 | 1.5 |

**Table 2.** Pilot results identifying spermatozoa from placing a drop of semen post-centrifugation (3000 x***g*** for 15 minutes) from a post-vasectomy sample on a glass slide and examined by direct light microscopy with a systematic x–y pattern over a 22x22 mm cover slip (BAS, 2002).

| | Number of samples | % |
|---|---|---|
| No sperm seen | 151 | 73.5 |
| ≥1 sperm | 18 | 8.8 |
| Total samples | 169 | |

**Table 3.** Pilot results identifying spermatozoa from placing a drop of semen from post-vasectomy samples on a large-volume (25 µL) fixed-depth glass slide and examined by direct light microscopy with a systematic x–y pattern.

| | 20 µm | % | 100 µm | % |
|---|---|---|---|---|
| No sperm seen | 172 | 84.3 | 142 | 69.6 |
| ≥1 sperm | 25 | 12.2 | 56 | 27.5 |
| Too many sperm to count | 2 | 1.0 | 3 | 1.5 |
| Motile sperm | 3 | 1.5 | 3 | 1.5 |
| Total | 204 | 100 | 204 | 100 |

## Materials and methods

An initial pilot trial was carried out examining 204 routine PVSA samples in a single laboratory using the BAS (2002) protocol and commercially available disposable, large-volume, fixed-depth slides (FDS) to establish if these could provide an equally accurate diagnostic method for the establishment of surgical success.

Disposable Leja (Nieuw-Vennep, The Netherlands) and Microcell (Vitrolife, Göteborg, Sweden) slides were supplied in two capacity sizes, one of 20 µm chamber height (3–5 µL volume; Leja/Vitrolife) and one of 100 µm chamber height (25 µL volume; Leja). The greater-depth chamber had been designed specifically to quantify low numbers of cells in suspension. During manufacture, all the glass used is washed and then coated to reduce cell adherence to the glass surface and prevent bubble formation in the chamber during filling.

Samples were collected by masturbation into wide-mouthed, non-toxic plastic containers. All samples were delivered by the patient, or his partner, within two hours of production. Other specimen acceptance criteria included i) collection of a complete specimen, and ii) abstinence from sexual activity for more than two days. Samples were examined within four hours of production to optimise detection of motile sperm. Hyperviscid samples, that failed to liquefy within one hour of production, were treated with type II salt-free lyophilised (>40 units/mg) α-chymotrypsin powder (from bovine pancreas; Sigma-Aldrich C4129-1G).

Prior to carrying out the standard BAS analysis (examination of a direct wet preparation, followed by examination of the centrifuged deposit), a 50 µL sample was removed from the well-mixed uncentrifuged sample and 3–5 µL placed in each of the small volume disposable slides (20 µm depth) and a 25 µL sample placed in the FDS (100 µm depth).[8] The slides were filled with a single continuous flow

**Table 4.** A comparison of each method in terms of the percentage of samples with sperm detected.

| | |
|---|---|
| Total samples | 204 |
| Positive by all three methods | 28 |
| Positive by BAS only | 4 |
| Positive by BAS and 20 µm | 3 |
| Positive by 100 µm only | 14 |
| Total positive BAS | 53 |
| Total positive 20 µm | 30 |
| Total positive 100 µm | 62 |

**Table 5.** Breakdown of results as a method comparison between the BAS-recommended technique and the use of the FDS for identification of sperm.

| | | BAS | | |
|---|---|---|---|---|
| | | Sperm seen | No sperm seen | Total |
| FDS | Sperm seen | 181 | 95 | 276 |
| | No sperm seen | 51 | 708 | 759 |
| | Total | 232 | 803 | 1035 |

**Table 6.** Breakdown of results as a method comparison between the BAS-recommended technique and the use of the FDS for population sensitivity.

| | | BAS | | | |
|---|---|---|---|---|---|
| | | Sperm seen | No sperm seen | | |
| | Sperm seen | 78.0% | 11.8% | → | Positive predictive value: 65.6% |
| FDS | No sperm seen | 22.0% | 88.28% | → | Negative predictive value: 93.3% |
| | | ↓ | ↓ | | |
| | | Sensitivity: 78% | Sensitivity: 88.2% | | |

and any excess removed once the fill was complete. The slide was allowed to settle for 30 minutes in a humid chamber and the entire area of each was examined using phase-contrast microscopy under x200 magnification.

The remainder of the original sample was examined by the standard BAS (2002) technique and by a different individual to the scientist examining the disposable slides. After consideration of the preliminary data, a three-centre trial was undertaken with the following laboratories taking part: Yeovil District Hospital (an andrology laboratory within the pathology department of a district general hospital); Hammersmith Hospital (Imperial Healthcare) (an andrology laboratory within the pathology department of a large London teaching hospital); and Nottingham University Hospitals NHS Trust (an andrology department as part of the fertility service in a large regional teaching hospital).

The protocol was to examine as many routine post-vasectomy samples as possible using the BAS method and the 100 µm (FDS) slides. Examination procedures were as for the trial period.

## Results

During the initial trial, the smallest capacity chamber (3 µL) showed a lower sensitivity than other methods so was not used in the full trial. The following results were obtained using direct microscopy according to the BAS (2002) method. Those samples where no sperm was seen on direct examination had a centrifuged deposit examination (Tables 1–3). The 20 µm slides had a demonstrable lack of sensitivity, with the error probabilities being 19.2% (95% confidence interval [CI]: 13.8–26.1%) compared to 2.1% for the 100 µm slide (95% CI: 0.5–6.5%). As this difference was statistically significant ($P<0.05$) it was decided that the smaller volume slide was not to be included in the larger study.

Overall the final comparison proved that the 20 µm slide had the poorest sensitivity when compared to the BAS method and the 100 µm slides, although all methods had some variance in detection of sperm (Table 4)

**Table 7.** Results of 50 repeat measures for sperm detection of slides prepared from a diluted UK NEQAS (Reproductive Science scheme[8]) sample based on a stock giving a theoretical value of 1000 sperm per mL.

| | |
|---|---|
| ~100 sperm per mL | Sperm recovery in 100% of slides |
| ~50 sperm per mL | Sperm recovery in 46% of slides |

*Muticentre study*

A total of 1104 patient samples were examined over the study period. Due to uncontrolled anomalies, 69 patients were excluded from the study. In addition to the examination methods, the total examination time was recorded for both methods in an attempt to provide manpower costing for both techniques.

In this type of study, the negative predictive value and specificity are the primary variables. Table 7 shows the complement of the negative predictive value which represents the probability $P^1$ of an error, where the sperm count of a patient is 0 according to the FDS slide, but where BAS still detects a positive sperm count. The complement of the specificity represents the probability $P^2$ of an error where the sperm count of a patient is zero according to BAS, but where the FDS slide still detects a positive sperm count. These error probabilities are $P^1=51/759$ (6.7%) and $P^2=95/803$ (11.8%), respectively. The negative predictive value is larger than the specificity, or equivalently $P^1<P^2$, which means that the sperm detection with FDS slides is slightly more sensitive than detection by examination using the BAS guidelines methodology.

In addition to the sensitivity issue, there was a distinct advantage in total examination times for the FDS slides. Although this was only established in one of the three units, the mean duration of examination time for the FDS slides was 7.69 minutes, with a standard deviation (SD) of 1.97 minutes. The averages for the BAS method were 13.63 minutes (SD: 4.37 minutes). Timings ignored any period that was dedicated to centrifugation or chymotrypsin incubation in the case of hyperviscid samples. The major problems that affected the BAS times were two examination phases and the presence of non-sperm cells in the deposit. Invariably, the FDS slides could be examined even with relatively high numbers of other cells present.

The study also attempted to establish detection levels of the 100 µm slide based on appropriate confidence limits.

A sample of known concentration was provided by the UK NEQAS Reproductive Science scheme[8] that had been distributed and where the returns showed little variance from the designated value. This sample was diluted to achieve a level of 1000 sperm/mL. This preparation was used as the stock solution to establish sensitivity levels of the slides. Multiple examinations showed that at probable concentrations of 100 sperm/mL there was sperm presence in every repeat (50 repeats); however, once levels reached 50 sperm/mL, 23/50 repeats showed that no sperm were detectable. The detection limit is defined as the smallest value that can be distinguished from zero with a

defined degree of confidence, and it is not possible by analysis to conclude that a substance is completely absent from a sample. For this reason, it is usual to determine the detection limit by extrapolating the imprecision at a very low concentration to zero; however, the present results agreed with the findings of Cooper *et al*.[7]

## Discussion

Using a small preliminary investigation followed by a larger multicentre trial, this study demonstrated the sensitivity and specificity of an alternative PVSA protocol employing a large, fixed-volume slide and compared this with the current best practice. Although the large-volume slide method did not incorporate a centrifugation step, its sensitivity was found to be slightly superior to the current recommended standard (BAS, 2002) method. A time-and-motion study showed financial benefit in avoiding the centrifugation step in favour of the large-volume slide method despite increased consumable costs.

Semen evaluation is complex and standardisation is difficult. For example, the first large-scale testing programme for clinical andrology laboratories in the United States reported that the inter-laboratory coefficient of variation (CV) for manual sperm concentration determination was 80%, with a range for one semen sample of 3–492 x $10^6$/mL.[9] Some of this difference is likely to be attributable to the use of various types of counting chamber, and there is considerable disagreement regarding the relative accuracy and precision of individual chamber formats. This variability among laboratories in establishing sperm numbers (and indeed other sperm parameters), especially with oligozoospermic samples such as those from PVSA, continues to contribute to the uncertainty in the clinical interpretation of analysis results.

Additional problems arise with samples exhibiting high viscosity and the presence of non-sperm cells and other detritus that is deposited during the centrifugation stage used in the BAS method. The FDS cells were routinely less affected by these problems and in general not significantly affected by any Segre-Silberberg (S-S) effect.[10] In principle, all capillary filled slides are affected by the Segre-Silberberg effect, which causes the transport of cells to the filling front during the flow of sample into the chamber. This can cause underestimation of the number assessed if only the central area of the analysis chamber is examined.

When using these disposable slides for counting normal semen samples, the dimension of any correction factor for the Segre-Silberberg effect will depend on many variables, such as chamber height, surface properties of the chamber, surface tension, flow velocity and viscosity. All of which combine to influence a full Poiseuille flow to the chamber. As all the variables in FDS chambers are kept constant during manufacture, the only variable that will affect the S-S effect is sample viscosity. In practice, when used for the evaluation of PVSA samples by examining the entire chamber, the S-S effect is less significant although the viscosity of the sample must be addressed. The distribution of sperm in post-vasectomy semen samples is not homogenous and thus highly viscous samples should be treated with a digestive enzyme to attempt to obtain even distribution of any sperm throughout the specimen.

Theoretically, the only issue with FDS cells is the depth of the chamber. If x200 magnification is used (x10 eyepiece, x20 objective), the focal plane of the microscope is 16–18 µm. Non-motile sperm cells will, after a few minutes, settle on the basal glass plane, allowing easy recognition and counting. At a depth of 100 µm there is an additional step where the objective will have to be racked through the sample to ensure motile sperm are not missed. In practice, this made no difference to detection comparisons between the two methods.

The measurement of examination times allowed accurate cost comparisons to be established. Final costings included full laboratory staff and on costs (i..e., taxes, pensions, national insurance), laboratory set up (i.e., making appointments, booking samples, consumables, wet preparation, deposit examination, typing, printing and validation of reports). The authors also included allowances for sperm vitality (assuming vitality carried out on 10% of samples), samples with motile sperm present (expanded semen analysis on 1% patients), and miscellaneous costs/year that covered internal quality control, decontamination, cleaning, apportioned maintenance contracts, stock control, patient information leaflet production, training, stationary, non-laboratory consumables, safety/waste disposal, secretarial costs, 'no show' administration, and chymotrysin use. Although the precise cost is hard to define at any given moment in time, especially when bearing in mind the different institutions and grades of staff that may perform these tests, it was shown that the FDS method was more cost-effective. The additional staffing requirement when following the BAS method gave an approximate 13% higher cost overall, even when the high consumable costs were taken into account.

There is always 'uncertainty' associated with any laboratory test; however, this three-centre trial has shown that PVSA can be performed more rapidly and more cost-effectively without examining both an initial wet preparation and a centrifuged pellet. Centres must be aware that best practice should be based on the assessment of risk and the laboratory method is not the only area of the PVSA process that has to be considered. Alongside the laboratory examination, the risk associated with specimen collection, transport, patient identity and accurate reporting of the result must also be assessed. Moreover, laboratories should consider a confirmatory second PVSA to be a sensible risk control measure or precaution against giving a patient clearance prematurely. For this reason, the recently revised recommendations from the Association of Biomedical Andrologists (ABA)[11] should be taken into account when defining the entire PVSA process, regardless of the laboratory method used to achieve the PVSA result.

In conclusion, the large-volume slides gave results that were at least as effective as the previously considered 'gold standard', and the two can be considered 'interchangeable'. It may be concluded that this method is an acceptable alternative to that described in the BAS 2002 guidelines and that cost considerations are an additional benefit. □

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